RESEARCH ARTICLE

Synthesis and Characterization of Pure and Capped Hydroxyapatite Nanoparticles

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ABSTRACT: Hydroxyapatite (HAp) is a well known biomaterial applied in orthopaedic and dental application as bone implants. Bone can easily be infected by microorganisms. In the present study, HAp nanoparticles are capped with *Kedrostis foetidissima* leaf extract (KHAp) by wet chemical assisted microwave irradiation (method) using calcium hydroxide as calcium source and orthophosphoric acid as phosphorous source. The synthesized samples were characterised by XRD, FT-IR, UV, SEM, EDAX and PL. Further in-vitro analysis was performed using gram positive bacteria *Staphyllococcus aureus* and *Bacillus subtilis* and Gram negative *Pseudomonas aeruginosa* and *Escherichia coli, candida albicans* fungi. The results of X-ray diffraction (XRD) analysis show the crystalline size of 20.04 nm for pure HAp and 16.71 nm for KHAp. FT-IR results confirm the presence of hydroxyl (O-H) and phosphate (PO_4^{3-}) groups. The results of UV and PL analysis reveal the band gap energy of 4.06eV. The emission band of KHAp is at 385.91nm. SEM and EDAX predict the spherical shaped morphology with proper proportion of 1.65. In-vitro analysis reveals the significant zone of inhibition for both bacteria and fungi.

KEYWORDS: Hydroxyapatite, *Kedrostis foetidissima,* capping, Antimicrobial activity, XRD, SEM

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1. INTRODUCTION

Hydroxyapatite (HAp) is a mineral form and naturally occurring material. The formula of calcium apatite expressed by $Ca_5(PO_4)_3(OH)$, but usually in $Ca_{10}(PO_4)_6(OH)_2$ to indicate that the crystal unit cell consists of two establishments. HAp is an adapted form of human bone, known as bone mineral. Carbonated calcium-deficient HAp is composed with dental enamel and dentin. HAp crystals initiate small calcium depositions, within a pineal gland and other structures. The mass of dentin and enamel in teeth have HAp in the range of 70 to 80%. [1]. HAp is widely used in medicine as filler and coating on bone and dental implants. [2]

HAp is used as substitute material for bone and tooth; it reduces the pain of bone during growth. It forms an appetite layer on its surface and combines with surrounding bone [3].

Green synthesis is a plant-mediated synthesis containing plant extract, flowers and fruits. Varieties of methods are used in perfect synthesis the NPs at ambient temperatures; neutral pH, low costs and environmental friendly manner [4]. Nanomaterials are synthesized by using various ways surrounded by biological alternatives, plants and plant extracts. They are cost efficient and require low maintenance. Hydroxyapatite in combination with antibiotics is very useful for the development of biomaterial for filling bone defects. [5]

Kedrostis foetidissima (Fig.1.1) is a traditional herb belonging to the family Cucurbitaceae. The edible parts of the plant are tubers, rhizome and stem. These parts are consumed by tribal people. This is an ethnomedical plant. So it is used for treating aliments, common cold, diarrhea, and measles. The phytochemical study of petroleum ether extract of leaf and chloroform extract of stem exposed the occurrence of flavonoids, triterpenoids, phenols, steroids, and glycosides. [6].

2. MATERIALS AND METHODS

Hydroxyapatite were prepared by using Calcium hydroxide [$Ca(OH)_2$] and orthophosphoric acid [H_3PO_4] as host precursors. The chemical reagents like NaOH solution, acetone and distilled water were used. Analytical reagent grade (99% purity procured from Sigma Aldrich).

2.1 Synthesis of Pure HAp:

1M of Calcium hydroxide is dissolved with 50ml of distilled water. 0.6 M of orthophosphoric acid is dissolved in 50ml of distilled water at room temperature. Both the solutions are stirred for half an hour separately. Then ortho phosphoric acid was added drop by drop into calcium hydroxide solution at room temperature. NaOH solution was added drop wise to maintain the pH of 12. This mixture was continuously stirred for three hours. The precipitate was aged for 24hrs. Thus the settled precipitate was washed with double distilled water and the final product was kept in microwave oven at 350 W for 30 minutes. The powder was calcined at 600°C in muffle furnace. The dried samples are grained in a mortar to get pure HAp nano powder.

2.2 Preparation of Leaf extract

The healthy leaves Kedrostis *foetidissima* were collected. The collected leaves were washed using double distilled water to remove dust particles. About 50g of the fresh leaf was boiled in 200ml of distilled

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water for 1 hrs. Then the leaf extract was filtered using No.1 whatman filter paper. The pH of leaf extract was around 7.

Fig 1.1: Kedrostis foetidissima leaf

2.3 Synthesis of KHAp

Kedrostis foetidissima leaf capped hydroxyapatite (KHAp) is prepared by using Leaf extract as solvent. 1M of Calcium hydroxide is dissolved in 50ml of leaf extract. 0.6 M of orthophosphoric acid is dissolved in 50ml of leaf extract at room temperature. Both the solutions are stirred for half an hour separately. Then ortho phosphoric acid was added drop by drop into calcium hydroxide solution at room temperature. NaOH solution was added drop wise to maintain the pH of 12. This mixture was continuously stirred for three hours. The precipitate was aged for 24hrs. Thus the settled precipitate was washed with double distilled water and the final product was kept in microwave oven at 350 W for 30 minutes. The powder was calcined at 600^{o}C in muffle furnace. The dried samples are grained in a mortar to get KHAp nano powder.

3. CHARACTERIZATION TECHNIQUES

3.1. XRD Analysis

XRD analysis is used to determine the crystalline nature and phase identification of the nanoparticles. The crystalline size of the particle was defined from Debye -scherrer's formula $D=K\lambda/\beta \cos \theta$. (3.1), Where, λ- wavelength of XRD, β- full width half maximum, θ- Bragg's angle.

The lattice parameter calculated by equation of $1/d^2= (4(h^2+hk+k^2)/3a^2)+(l^2/c^2))$ Where, d is the plane spacing, a and c are the lattice parameters which confirms the hexagonal structure. The unit cell volume (V) of the sample was described using the given equation: $V= (\sqrt{3}/2) + a^2+c^2$ [7]

3.2. FT-IR Spectroscopy

Fourier Transform Infrared Spectroscopy or FTIR Spectroscopy is a systematic method used to recognize polymeric, organic and inorganic materials in the region of 4000-400 cm^{-1}. In this method the infrared light is used to scan the test samples and observe chemical properties of the sample [8].

3.3. UV-Vis Spectroscopy

Ultraviolet-visible spectroscopy is an important analytical tool for chemistry. This is the most frequently used techniques in chemical laboratories. This tool is used for the qualitative study and classification of chemicals. On the other hand, it is used for quantitative determination of different organic and inorganic compounds in solution. This method is mainly used to measure transmission or absorption in liquids and transparent or opaque solids.[9]

3.4. SEM and EDX Analysis

SEM analysis is used for elemental analysis, mineral orientation, morphology and contrasts [10]. EDAX is a systematic technique for elemental analysis or chemical composition of a sample.

3.5. Photoluminescence spectroscopy [PL]

Photoluminescence spectroscopy is a contactless, flexible, powerful optical method of searching the electronic structure of materials. Used to determination of Band Gap, Impurity Levels and Defect Detection, Material Quality, Molecular structure and crystallinity. The photoluminescence spectrum of the as-prepared sample was obtained over a range 300–800 nm using RF- 5301 Fluorescence spectrophotometer equipped with a 150W Xenon lamp as the excitation source. [11].

3.6. Antimicrobial Activity

Antibacterial activity of aqueous and solvent extracts was determined by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). The tested bacteria were spread on nutrient agar plates. The wells of 8 mm diameter were punched into the agar medium and filled with 100 µl (25 mg/ml) of plant extract and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 37° for 24 h. Wells containing the same volume of DMSO (10%), hexane, chloroform, methanol, ethanol and distilled water served as negative controls while standard antibiotic discs of imepenem (10 µg) and vancomycin (30 µg) were used as the positive controls. After incubation, the diameters of the growth inhibition zones were measured in mm. Three replicates were carried out for each extract against each of the test organism. Data were expressed as mean ± standard deviation [12].

4. RESULTS AND DISCUSSION

The prepared nanoparticles were characterized by various techniques like X-ray diffraction (XRD), Fourier transform infra red spectroscopy (FT-IR), UV- Vis spectroscopy, scanning electron microscopy(SEM), energy dispersive X-ray spectroscopy (EDAX), Photo luminescence (PL) analysis and antimicrobial activity.

4.1. XRD Analysis

The XRD pattern of prepared HAp and KHAp were shown in Fig: 4.2.The prepared sample confirms the presence of hexagonal structure and it is well matched with JCPDS file No: 09-0432. The broad diffraction peaks of HAp and KHAp at $2\theta=42.13^{0}$, 54.37^{0} and 75.49^{0} are clearly deducted. The indexed hkl planes are (302), (104) and (602). No impurity peaks were detected. The average crystalline size (D)

of HAp and KHAp was 20.04 and 16.71nm. Thus the average crystalline size of KHAp gets reduced due to incorporation of leaf extract. The presence of polyphenols and tannins acts a reducing agent to reduce the crystalline size of nanoparticles. The unit cell volume (V), lattice parameters a and c decreased due to increase in crystalline size and were shown in table 4.1.

4.2. FT-IR Spectroscopy

The FT-IR spectra predict the characteristics bands present in the sample. The presence of phosphate and hydroxyl groups are identified which corresponds to HAp structure. The FT-IR spectrum of HAp shows the vibration modes of phosphate at 870.70, 570.82 and 1044.26cm^{-1}. Hydroxyl groups of HAp are revealed at 3454 cm^{-1} and 3744 cm^{-1}. The vibration mode at 569.86, 872.631 and 1043 cm-1 reveals the presence of phosphate group and Hydroxyl groups at 3456.78 cm^{-1} and 3636.12 cm^{-1} for KHAp. Peaks at 1421.28 cm^{-1} and 1420.32 cm^{-1} represents the CH_3 stretching of carboxylic acid. The results were reported in Fig 4.3 and table 4.2.

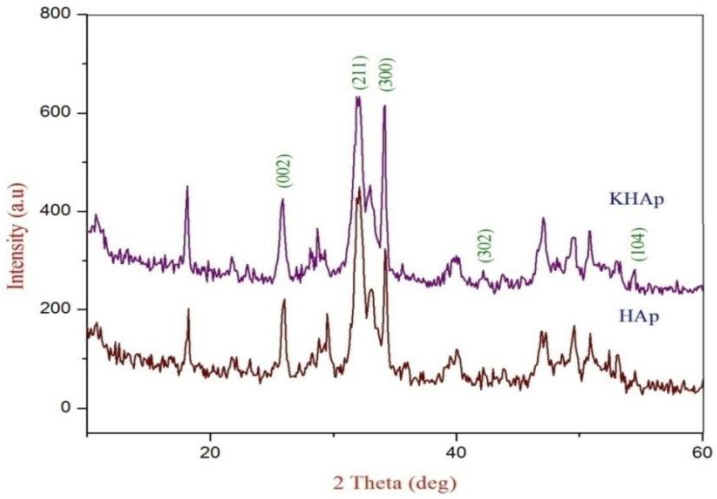


Fig: 4.2. XRD pattern of the HAp and KHAp.

Table 4.1. XRD paramaters of HAp and KHAp

Sample name	FWHM (deg)	d (Å)	Intensity (Counts)	Crystalline Size (nm)	hkl	Lattice Constant		Unit Cell Volume (Å^3)
						a=b	C	
HAp	0.68 0.38 0.40	2.133 1.684 1.258	12 7 9	12.45 23.29 17.12	302 104 602	9.32	6.89	518.57
KHAp	0.52 0.46 0.69	2.142 1.686 1.258	12 14 9	6.20 19.40 14.55	302 104 602	9.4	6.8	530.29

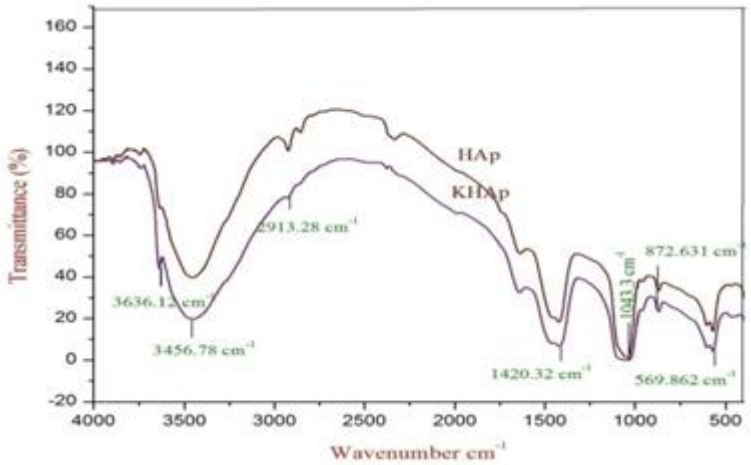


Fig: 4.3. FT-IR pattern of the HAp and KHAp.

Table 4.2. Functional groups of HAp and KHAp

S.No	Sample Name	Wave number (cm^{-1})				
		O-H Stretching Vibration	C-H Stretching Vibration	C=C Stretching Vibration	CH_3 Bending Vibration	P-O Stretching Vibration
1	HAp	3454.85	2925.48	1633.98	1432.28	1044.26
2	KHAp	3456.78	2913.28	1629.92	1420.32	1043.3

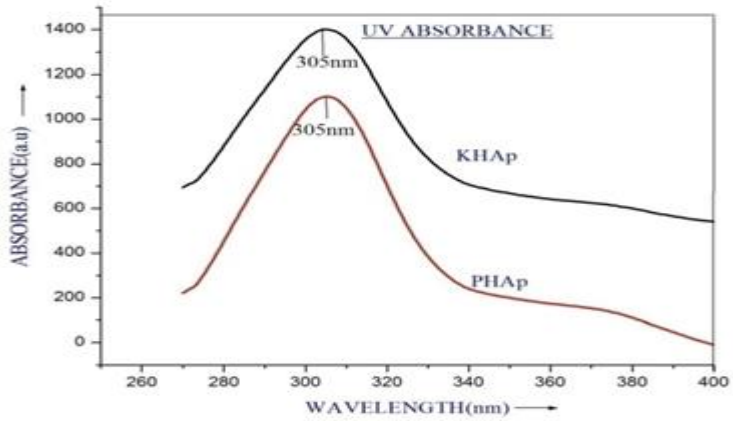


Fig: 4.4. UV –Vis spectrum of HAp and KHAp

4.3. UV Analysis

The optical properties and band gap energy of the samples are investigated using UV visible spectroscopy. The absorption spectra of HAp and KHAp nanoparticles are found in the wavelength of 305nm in Fig 4.4. The band gap energy of HAp and KHAp was calculated as 4.06 eV. Both the sample has same wavelength and band gap energy due to quantum size effect and electronic structure modifications.

4.4. SEM and EDX Analysis

The SEM analysis is used to study the morphological structure of the prepared nano composites. The synthesized samples of HAp and KHAp nanoparticles were crystalline in nature. Fig: 4.5 HAp (a) shows the agglomerated nano structure and KHAp(b) reveals the small spherical like nano structure. The particle size of the HAp was 34nm to 83nm in diameter and KHAp was 35 to 61nm in diameter.

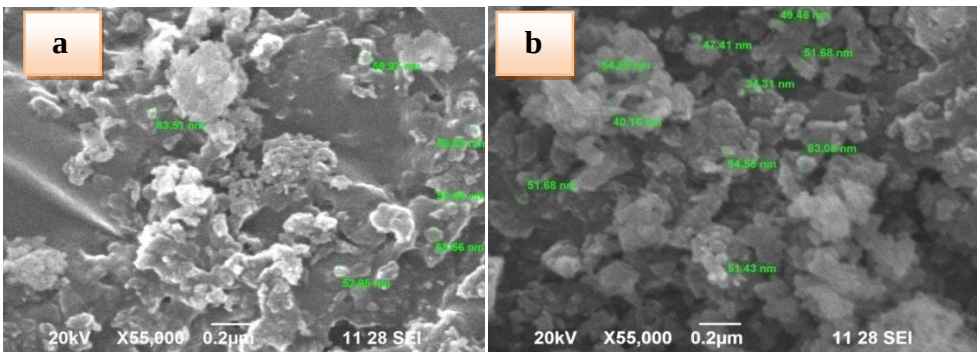
The results are reported in Fig 4.5(c,d). The functional groups of Ca, P and O are present in the sample. It confirms the purity of the composition elements. The atomic weight of Ca (12.63%) and P (7.51%) for HAp. The atomic weight for Ca in KHAp was 11.12%, and P was 6.66% respectively.

4.5. Photoluminescence Spectroscopy

The intensity of emission radiations was absorbed by Photoluminescence spectroscopy.The emission radiation of HAp and KHAp are shown in Fig:4.6 The excitation wavelength occours at the range of 228.34nm for both HAp and KHAp .The emission band of KHAp located at 385.9nm. In HAp, there was no shift present at the range.the intensity peaks increased. Due to the addition of leaf extract. The standard band gap energy of HAp around the range 3.45 eV to 5.40 eV ,while The calculated band gap energy of both the samples are 4.06 ev.

4.6. Antimicrobial Activity

The anti bacterial activity of pure HAp and KHAp was studied against gram positive bacteria of *B.subtilis* (NCIM 2063) and *S.aureus* (NCIM 2079), Gram negative bacteria of *P.aeruginosa* (NCIM 2000) and *E. coli* (NCIM 2065) by agar well diffusion method. The antifungal activity was investigated against *C.albicans(NCIM 3100).*



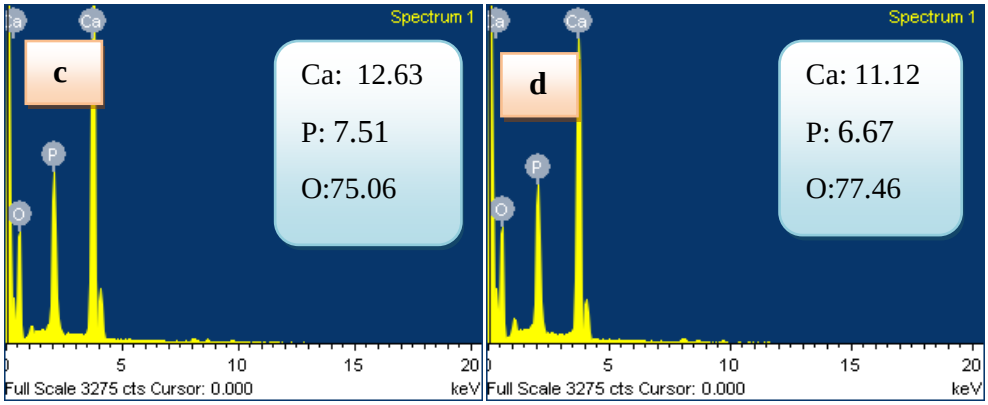


Fig.4.5. SEM and EDX analysis for HAp and KHAp

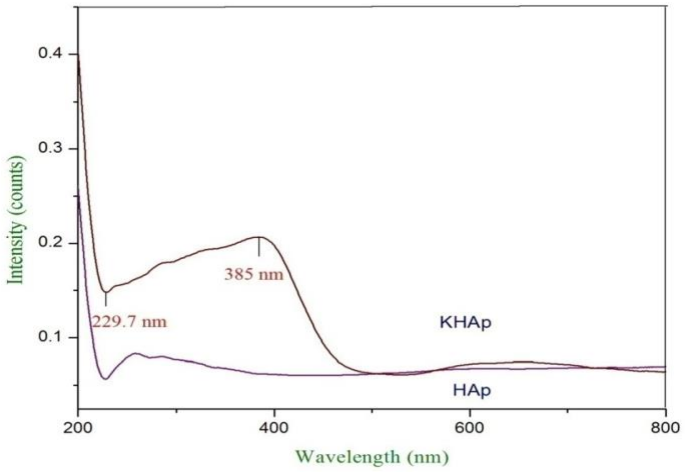


Fig:4.6.Photoluminescence spectra of HAp and KHAp

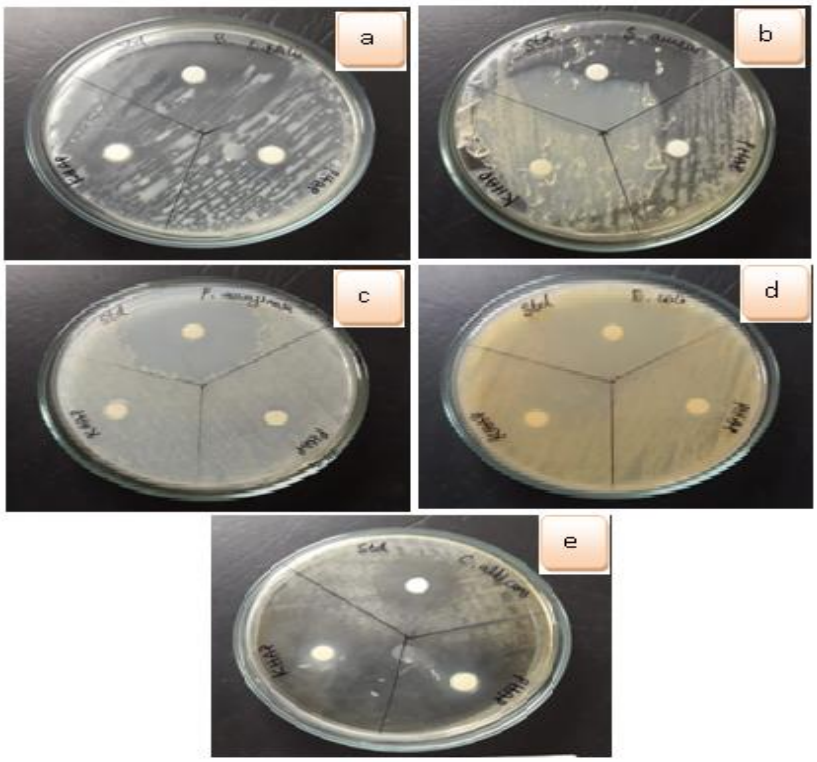


Fig : 4.7. Photographs of antimicrobial results of HAp and KHAp for Gram positive bacteria *B.subtilis* and *s.aureus*, Gram negative bacteria *P.aeruginosa* and *E.coli* and fungi *C. albicans*.

Table: 4.3. zone growth inhibition related to HAp and KHAp nanoparticles for Gram positive bacteria *B.subtilis* and *S.aureus*, Gram negative bacteria *P.aeruginosa* and *E.coli* and fungi *C.albicans*.

S.No	Sample name	Zone of inhibition (mm)				
		Gram positive		Gram negative		Fungi
		B.subtilis	*S.aureus*	*P.aeruginosa*	*E.coli*	*C. albicans*
1.	**HAp**	12	08	10	08	13
2.	**KHAp**	09	08	09	-	15

The antibacterial and anti fungal activity of of pure HAp and KHAp are displayed in Fig:4.7 and the values are Tabulated in Table. 4. The zone of inhibition for gram positive bacteria against *B.subtilis* and *S.aureus* was 12mm and 8mm for HAp. In KHAp the zone of inhibition was 10mm and 8mm respectively. The gram negative bacteria of *P.aeruginosa* and *E.coli* have a Zone of inhibition of 10mm and 8mm for HAp. In KHAp *P. aeruginosa* have a Zone of inhibition of 9mm and there was no inhibition absorbed in E.coli. This is due to mycelia growth in E.coli bacteria. Ciprofloxacin was a standard positive control used for bacterial strains and fluconazole for fungi. The prepared KHAP samples are effective when compared with HAp. The antifungal activity shows significant zone of inhibition for KHAp than HAp. Table:4.3 shows the antimicrobial activity of prepared sample.

The significant ZOI values ranging from 8mm to 15mm was revealed. The fungi *C.albicans* shows the highest ZOI of 15mm and the gram positive bacteria *E.coli* shows lowest ZOI of 8mm. No ZOI was revealed for KHAp against *E.coli*.Both the samples showed significant ZOI for gram positive bacteria.

The amide bands and proteins are present in the functional groups because of leaf extract [13]. The SEM image of synthesized HAp and KHAp reveals the size of the nanoparticle. EDX explains the elemental composition and purity of the sample [14].

The leaf extract of *Kedrostis foetidissima* is an effective remedy for complications arising from the measles virus infection in children. Both HAp and KHAp was active against gram positive and gram negative bacterial species of *B.subtilis*, S.*aureus, and P. aeruginosa, E. coli and* fungi *c.albicans* . The antimicrobial activity of KHAp was significant in both the samples with and without leaf extract. Hence both the samples can act as a chemo preventive agent and antioxidant. [15].

6. CONCLUSION

Hydroxyapatite nanoparticles were synthesised by green method and the samples were prepared by facile microwave irradiation method. The XRD pattern revealed the crystalline size, lattice parameter and unit cell volume of the sample. The crystalline size reduced in KHAp compared to HAp. The FT-IR spectrum confirms the presence of functional groups. SEM analysis predicts the spherical shaped morphological structure. EDAX analysis confirms the presence of calcium and phosphate groups in the ratio of 1.65. The observed band gap energy was 4.06eV. Antibacterial and antifungal activity predicted the zone of inhibition. Significant ZOI for gram positive bacteria was revealed. *E.Coli* shows no ZOI. The anti fungal activity shows good ZOI. Thus we conclude that green synthesized hydroxyapatite nanoparticles can be used as a best candidate for dental applications.

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